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Simultaneous Gene Editing by Injection of mRNAs Encoding Transcription Activator-Like Effector Nucleases into Mouse Zygotes

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Injection of transcription activator-like effector nucleases (TALEN) mRNAs into mouse zygotes transferred into foster mothers efficiently generated founder mice with heritable mutations in targeted genes. Immunofluorescence visualization of phosphorylated histone 2A (γH2AX) combined with fluorescence *in situ* hybridization revealed that TALEN pairs targeting the *Agouti* locus induced site-directed DNA breaks in zygotes within 6 h of injection, an activity that continued at reduced efficiency in two-cell embryos. TALEN-Agouti mRNAs injected into zygotes of brown FvB × C57BL/6 hybrid mice generated completely black pups, confirming that mutations were induced prior to, and/or early after, cell division. Founder mice, many of which were mosaic, transmitted altered *Agouti* alleles to F_1 pups to yield an allelic series of mutant strains. Although mutations were targeted to "spacer" sequences flanked by TALEN binding sites, larger deletions that extended beyond the TALEN-binding sequences were also detected and were similarly inherited through the germ line. Zygotic coinjection of TALEN mRNAs directed to the *Agouti*, *miR-205*, and the *Arf* tumor suppressor loci yielded pups containing frequent and heritable mutations of two or three genes. Simultaneous gene editing in zygotes affords an efficient approach for producing mice with compound mutant phenotypes, bypassing constraints of conventional mouse knockout technology in embryonic stem cells.

Genome editing can be used to alter the chromosomal DNA of mouse embryonic stem (ES) or induced pluripotent (iPS) cells, which, in turn, can generate live mice carrying the same genetic modifications throughout all tissues. Although targeted gene disruption via homologous recombination has provided an exceptionally powerful strategy for dissecting gene functions (1), the methods are cumbersome due to the low efficiency of correctly incorporating targeting vectors into defined chromosomal sites in ES cells, the demand for selection and screening strategies to identify properly targeted clones, the requirement for karyotyping to exclude defective aneuploid derivatives, and the need for time-consuming mouse husbandry to generate animals with multi-allelic mutations. Although inactivating genes by RNAi, even in inducible systems, provides an alternative approach (2, 3), these methods are limited by incomplete gene "knockdown," unpredictable off-target effects, and transient inhibition of target gene expression.

Novel technologies employing transcription activator-like effector nucleases (TALENs) have recently been introduced to modify the genomes of diverse organisms, including yeasts, worms, flies, plants, and various mammalian species (reviewed in references 4, 5, and 6), bypassing the low efficiency, time constraints, and high cost of conventional gene targeting strategies in mice. TALENs comprise a nuclease fused to a tailored, sequence-specific DNA-binding domain consisting of assembled TALE monomers (7–10). Each TALE monomer contains 33 to 34 amino acid residues with a repeat variable diresidue at positions 12 and 13 that provides single nucleotide recognition (11, 12). Multimerization of TALE monomers directs binding of the fusion proteins to specific DNA sequences of considerable length (each usually ~20 nucleotides). "Forward" and "reverse" TALENs, each containing a fused C-terminal endonuclease domain, such as FokI, can be directed to two sets of appropriately spaced sequences (usually 14 to 16 bp within cDNA strands), thereby providing stringent target recognition while allowing correctly juxtaposed FokI monomers to initiate targeted double-strand breaks (DSBs) precisely within the spacer region between the bound TALENs. The DSBs are repaired by inaccurate nonhomologous end joining (NHEJ) (13) to yield insertions and deletions, and they can also facilitate homologous recombination-directed DNA repair driven by oligonucleotides or conventional targeting plasmids (4–6). TALEN-directed genome editing has been successfully applied to human ES and iPS cells (14, 15) and, for example, to mouse ES cells to generate mice with targeted disruptions and insertions in two Y chromosome-linked genes (16).

Although TALENs can be targeted to nearly any DNA sequence of interest to efficiently modify target genes (17, 18), TALEN pairs must be individually assembled from TALE monomers for each target gene, making library construction difficult. Assembly of custom TALE arrays by "Golden Gate" molecular cloning is rapid and relatively straightforward (7). However, newly developed methods for TALEN assembly show greater promise in facilitating high-throughput gene editing (19–21), allowing the construction of highly complex TALEN libraries, including one spanning the entire human genome (22).

A newly applied approach to gene editing involves the injection

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of TALEN DNAs or RNAs into zygotes implanted into pseudopregnant females and used to derive founder animals in a single step. The methods have been used to modify single genes with variable efficiencies (6, 23–26), but consistent characterization of mutations in founder animals has suffered from ambiguities stemming from different protocols and methods of analysis. We now report the use of a highly efficient TALEN-based strategy for gene editing in mouse zygotes that yields a very high frequency of heritable mutations at the targeted sites. Mosaic founder mice derived from injected zygotes transferred into surrogate mothers transmit one of a panoply of different mutant alleles to their individual F_1 progeny. This system facilitates efficient and simultaneous editing of multiple target genes in founder pups, ultimately yielding individual F_1 offspring that unambiguously carry one of several different combinations of mutant alleles.

MATERIALS AND METHODS

TALEN design, assembly, and mRNA synthesis. Sequences of TALEN pairs targeting the *Agouti* initiation codon, pre-*miR-205*, and the *Arf* initiator codon (see Fig. S1 in the supplemental material) were compared against the whole mouse genome using the National Center for Biotechnology Information (NCBI) nucleotide BLAST search program to rule out other adventitiously targeted loci. TALEN monomers were assembled using a commercial tool kit (Addgene, catalog no. 1000000019) as described previously (27). Eighteen assembled monomers were inserted into one of four backbone plasmids, each of which included an N-terminal signal specific for thymidine and one of four base-specific C-terminal TALEs to generate a 20-nucleotide recognition sequence. These were fused in frame at the N terminus with three Flag tags and a nuclear localization sequence and at the C terminus to sequences encoding the FokI endonuclease. Correctly assembled TALENs cDNAs were verified by nucleotide sequencing analysis; transfection of cDNAs into human 293T cells using Lipofectamine 2000 (see pilot studies below), followed by immunoblotting of cell lysates with antibodies to the FLAG epitope (M2; Sigma-Aldrich), confirmed the expression of proteins of the expected masses (~135 kDa). TALEN coding sequences were released from the assembled vectors with EcoRI and SacI, and FspI was used to digest the vector fragment before ligating the TALEN-containing fragment into pBluescript SK(+). The plasmids were linearized with EcoRI and purified using a Qiagen PCR purification kit. Linearized plasmids (each 1 μg) were used for *in vitro* transcription using a T3 mMESSAGE mMACHINE Ultra kit (Ambion/Invitrogen) to yield capped and tailed transcripts. The mRNAs were extracted in phenol-chloroform-isoamyl alcohol (Invitrogen), followed by chloroform alone, and RNA in the aqueous phase was precipitated with isopropanol at −20°C. Pelleted mRNAs were suspended in 10 mM Tris-HCl (pH 7.6) containing 1 mM EDTA, quantified by spectrophotometry (A_{260}/A_{280} ratio), diluted to 50 ng/μl, and frozen at −80°C until used for microinjection.

Pilot studies with ES cells. The 129/SvJ ES cell line F12, derived by Jacqueline Surtel (St. Jude Children's Research Hospital) was routinely expanded on gelatin-coated culture dishes in ES cell medium as described previously (28). Wild-type F12 ES cells were plated into single wells of a six-well culture dish (Corning). For each TALEN pair, 5 μg of TALEN forward plasmid, 5 μg of TALEN reverse plasmid, and 1 μg of pEGFP-C1 reporter plasmid (Addgene) were suspended in 1 ml of Opti-MEM medium (Gibco) and transfected using 10 μl of Lipofectamine 2000 (Invitrogen) into single wells. Optimized transfection efficiencies were obtained 5 h after plating. Cells were treated with trypsin 48 h later and sorted by flow cytometry for green fluorescent protein (GFP) expression. Sorted cells were replated at a low density (1,000 cells/ml) on gelatin-coated 10-cm diameter dishes. Selected individual clones were dispersed by trypsinization into single cells by pipetting, half of which were taken for genotyping and sequencing and the rest for subsequent culture. Cells exhibiting targeted mutations were subjected to karyotypic analysis and frozen for future use. The frequencies of TALEN-induced mutations per number of GFP^+ transfected clones examined by nucleotide sequencing were 83% for *Agouti*, 52% for *miR-205*, and 79% for *Arf*. When suspended to form embryoid bodies (EBs), *Arf*-null ES cells exhibit a significant temporal delay in generating extraembryonic endoderm, a defect that is rescued by enforced overexpression of miR-205 (28). Similar delays in forming extraembryonic endoderm were observed in EBs containing biallelic TALEN-inactivated *Arf* or *miR*-205 genes, confirming the expected phenotypic consequences of gene inactivation.

We also cointroduced an *Arf-Cre* targeting vector containing host cell flanking sequences (29) into ES cells, together with the TALEN-Arf plasmids and a fourth reporter plasmid encoding enhanced GFP (EGFP). Cells transfected 5 h after plating and isolated 48 h later by fluorescence-activated cell sorting were expanded, and single-cell-derived colonies were subjected to nucleotide sequence analysis and Southern blotting 7 days later. Although no recombinants were detected among 33 clones from which TALENs were omitted, proper homologous recombinants were isolated in two of 42 clones when TALENs were included.

Mouse strains, genotyping, and nucleotide sequencing. FvB/N and C57BL/6 mice (Jackson Laboratories) were maintained in accordance with the St. Jude Children's Research Hospital's Institutional Animal Care and Use Committee, as required by the U.S. Animal Welfare Act and National Institutes of Health policy for proper care and use of laboratory animals for research. Genomic DNAs of single ES colonies or tail tissues of individual pups were extracted in 100 mM Tris-HCl (pH 8.3), 100 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 100 μg of proteinase K/ml. Samples were incubated for 8 h at 55°C and for 45 min at 85°C, and debris was removed by microcentrifugation for 10 min at 15,000 rpm. Genomic DNAs were amplified using primers listed in the supplementary methods in the supplemental material. PCR products were purified (Qiagen PCR purification kit) and subjected to Sanger sequencing using the indicated forward or reverse PCR primers for each gene. Homozygous mutations were determined by comparison to reference sequences downloaded from NCBI. Heterozygous mutations were determined by filtering out the known sequence of the wild-type allele. In some cases, ambiguous mixed sequence reads were resolved after TA subcloning (Promega) of PCR-amplified products and sequencing of at least five individual subclones.

Intranuclear and intracytoplasmic microinjection. Freshly fertilized 0.5-day-postcoitus (dpc) single-cell embryos (zygotes) were obtained from freshly weaned superovulated FvB/NHsd mice. Females were intraperitoneally administered 5 IU of pregnant mares' serum gonadotropin and, 46 to 48 h later, 5 IU of human chorionic gonadotropin (HCG) and mated with male C57BL/6J mice 1:1 overnight. Females with coital plugs the next morning were euthanized, the oviducts were surgically removed, the cumulus mass was isolated, and the eggs were released by nicking of the ampulla. Follicular cells were removed by enzymatic exposure in M2 medium with hyaluronidase (Sigma-Aldrich). Recovered zygotes were cultured until both pronuclei were microscopically visible, which was approximately 21 to 27 h post-HCG treatment. Approximately 150 to 200 zygotes were transferred to a microdrop of M2 medium on a microinjection slide overlaid with mineral oil, and the slide was placed on an inverted microscope. Microinjection pipettes were filled with either 5 or 50 ng of TALEN forward and reverse mRNAs/μl or with 3 ng of linear plasmid DNAs/μl (see Results and Table 1). For multiple gene targeting, the TALEN mRNA pairs were combined at a final concentration of 50 ng/μl each. Each embryo was held with light suction by a holding pipette and rotated until one of the two pronuclei was visible. The mRNA was injected into the cytoplasm and DNA into one pronucleus. Injection of a few picoliters of solution was monitored by the visible swelling of the pronucleus or presence of a jet stream in the cytoplasm, respectively. Microinjected zygotes were surgically transferred the same day to the infundibula of 0.5-dpc-pseudopregnant CD-1 foster mothers (20 to 25 zygotes per recipient). Surrogate mothers were prepared by mating female CD-1 mice in estrus with vasectomized B6CBAF1/J males 1:1, followed by the identification of coital plugs the next morning. Approximately 25

TABLE 1 Gene disruption efficiency after zygotic injection[a]

Injection and targeted gene	Dose (ng/μl)	No. of transferred zygotes	No. of pups	Total mutation frequency (% of pups)
DNA injection				
Agouti	3	72	9	0
Agouti	3	72	26	0
RNA injection				
Single TALEN pair: *Agouti*	50	18	6 (2 black)	67
	50	20	6 (1 black)	50
	5	52	11 (7 black)	82
Double TALEN pair				
Agouti + *miR-205*	50 each	26	13	85 (*Agouti*), 85 (*miR-205*)
Agouti + *miR-205*	50 each	22	3	100 (*Agouti*), 100 (*miR-205*)
Triple TALEN pair: *Agouti* + *miR-205* + *Arf*	50 each	250 (10 foster mothers)	43	83 (*Agouti*), 64 (*miR-205*), 46 (*Arf*)

[a] All TALEN pairs were tested for activity in pilot studies performed using Lipofectamine transfection of mouse ES cells, followed by expansion of individual colonies at 5 h posttransfection and Sanger sequencing using the same primers. The amino acid sequences of all TALEN targeting constructs are shown in Fig. S1 in the supplemental material. After zygote injection, groups of 18 to 26 zygotes were transferred into individual foster mothers that yielded the indicated numbers of founder pups for subsequent analysis. Primer-amplified tail DNAs extracted from F_0 pups were directly sequenced using primers flanking the individual TALEN target sites, as illustrated in the figures. To resolve ambiguities, some tail DNAs were TA cloned, and the nucleotide sequences of five individual clones randomly selected from each library were determined. After injection of three TALEN pairs, the tail DNAs of 25, 36, and 24 F_0 mice were analyzed for *Agouti*, *miR-205*, and *Arf* sequences, respectively. The 24 mice analyzed for *Arf* mutations were selected from those already determined to have mutations of the other two genes.

embryos were placed into one oviduct of each 0.5-dpc surrogate mother with a transfer pipette via the infundibulum. Full-term pups were obtained by natural labor about 19 days later.

Embryo γ-H2AX immunofluorescence and FISH. Cultured embryos were studied at 6 and 24 h after Agouti-TALEN injection. Removal of zonae pellucidae and mounting were performed as described previously (30), followed by sequential immunofluorescence and DNA fluorescence *in situ* hybridization (FISH). Fixed slides stored at −20°C in 70% ethanol were rehydrated in phosphate-buffered saline (PBS) for 1 min, followed by blocking in 0.3 M NaCl–30 mM trisodium citrate (2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]) containing 1% bovine serum albumin for 5 min at room temperature. The slides were incubated for 45 min at room temperature in blocking buffer containing a 1:1,000 dilution of mouse antibodies directed to serine-139 of γ-H2AX (clone JBW 301; Millipore). After a 5-min wash in PBS, the slides were incubated for 45 min in blocking buffer containing a 1:100 dilution of goat anti-mouse immunoglobulin G conjugated to Alexa Fluor 488 (Life Technologies). Slides washed in PBS were fixed for 10 min at room temperature in PBS containing 4% paraformaldehyde, 0.5% Tween 20, and 0.5% Nonidet P40 and, after another wash, treated on ice for 10 min with 0.2 N HCl and 0.5% Triton X-100. Rinsed slides were treated with 70% formamide–2× SSC for 10 min at 80°C, followed by dehydration in an alcohol series consisting of 70, 80, and 100% ethanol for 2 min each. Slides were hybridized with a bacterial artificial chromosome (BAC) FISH probe in 50% formamide, 10% dextran sulfate, and 2× SSC. The probe was prepared by nick translation with Alexa Fluor 594-dUTP (Molecular Probes) of a BAC cloned from the *nonagouti* locus, RP23-331P9.

Wide-field fluorescence microscopy was performed with a Nikon E800 microscope with the images being acquired through a 60× plan apochromatic objective with a numerical aperture of 1.4 and a Photometrics Cool Snap ES camera. Nikon Nis Elements software version 4.2 was used to capture and process the images. A multi-band-pass filter set (Chroma Technologies), housed in a single filter cube, contained the multi-band-pass dichroic mirror and emission filter, together with excitation filters, with a filter wheel controlled by the software. The microscope camera captures a picture of each color independently. The microscope moves the stage in a defined increment in the *z* dimension, capturing sets of images of each color and continuing until the range of *z* dimensions has been completed. This system assures absolute fidelity with regard to the spatial relationships among the various colors, and the software reconstructs the many component images into a single composite image. A determination of colocalization of signals was made if γH2AX foci and FISH signals were contiguous or overlapping in the *x*, *y*, and *z* dimensions.

RESULTS

TALEN-induced mutation of the *Agouti* gene in zygotes. In order to easily identify TALEN-targeted mice, we chose to disrupt the color determination gene *Agouti*, which acts within hair follicles to regulate coat color pigmentation (31). The wild-type *Agouti* locus (denoted *A/A*) is not penetrant in albino FvB/N mice which lack tyrosinase. In contrast, C57BL/6 (*Tyrosinase*$^{+/+}$) black mice have an ~11-kb DNA insertion in *Agouti* intron 1, which inactivates the locus (denoted *a/a*, formally named *Nonagouti*, MGI:87853 on mouse chromosome 2). Hybrid FvB/N × C57BL/6 heterozygotes (*A/a*; *Tyrosinase*$^{+/-}$) are agouti (brown). Plasmids encoding forward and reverse TALEN-Agouti recognition proteins were engineered to contain three N-terminal tandem flag repeats, a nuclear localization signal (NLS), 20 ordered monomer TALE peptides (the first TALE recognizing T), and a C-terminal FokI nuclease (Fig. 1A), which cleaves targeted DNA when dimerized (Fig. 1B). In pilot experiments, different pairs of TALEN-Agouti forward and reverse vectors were transfected into mouse ES cells to determine the frequency of generated mutant alleles (summarized in Materials and Methods). An optimally efficient TALEN pair (Fig. 1B; see Fig. S1 in the supplemental material) induced mutations in 40 of 48 independently derived ES cell clones and was chosen for further study.

We microinjected TALEN-Agouti forward and reverse plasmid DNAs linearized by EcoRI at 3 ng/μl into the pronuclei of mouse zygotes and transferred ~24 into each of six foster mothers. Of 35 pups born, none were black, and all retained their wild-type "*A*" and "*a*" alleles (Table 1). In contrast, after the transfer of TALEN-Agouti sequences into pBluescript SK(+) and transcription of mRNAs from the T3 promoter, microinjection of purified mRNAs into the cytoplasm of hybrid FvB/N × C57BL/6 zygotes yielded completely black pups (Fig. 1C and Table 1), suggesting that TALEN mRNAs generated mutations in the wild-type "*A*" allele prior to or soon after

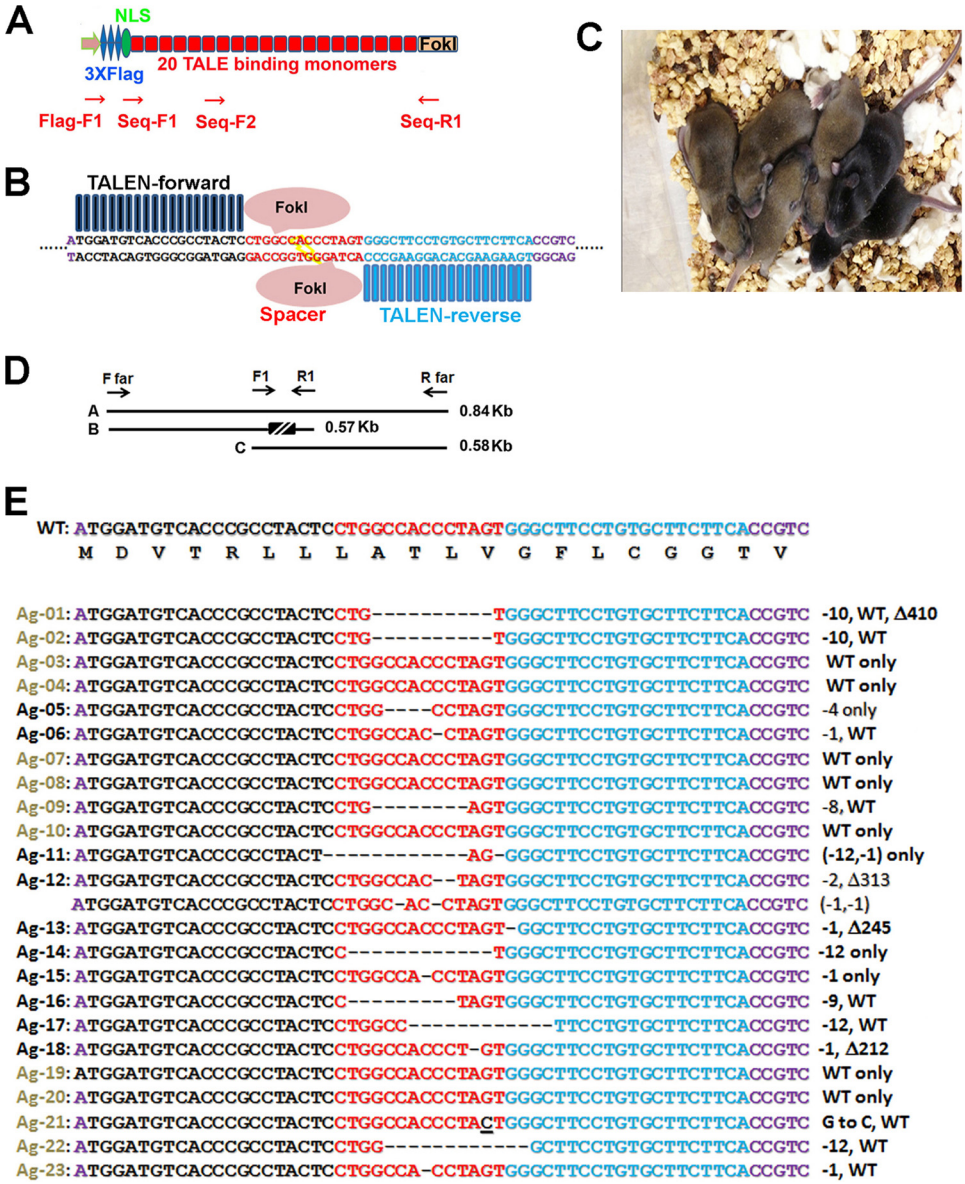

FIG 1 Generation and genotyping of founder mice produced by TALEN-Agouti mRNAs. (A) Schematic drawing of TALEN plasmids containing three N-terminal Flag epitopes, a nuclear localization signal (NLS), 20 TALE modules, and a fused C-terminal FokI nuclease. The positions of primers used for sequence verification of the targeting plasmids are indicated below the figure. Plasmids transfected into 293T cells encoded anti-Flag precipitable proteins of predicted masses. (B) Schematic illustration of binding of forward (black) and reverse (blue) TALEN pairs separated by 14-bp spacer sequences (red) at the *Agouti* locus. Host flanking sequences, including the "A" of the initiation codon (left) are shown in purple. (C) Litter of six F_0 pups (two black) generated from a foster mother implanted with zygotes injected with Agouti-TALEN pairs. (D) Primers used for PCR amplification and nucleotide sequencing of tail DNAs of F_0 mice; the box indicates sequences encoding TALEN binding sites and flanking sequences between primers F1 and F2. (E) Nucleotide sequences of tail DNAs from TALEN-Agouti (Ag) F_0 mice; the color code is identical to that shown in panel B. Black and brown pups are indicated by the corresponding Ag font colors (left). Mutated alleles detected in each F_0 mouse are summarized at the right. Symbols: WT, wild type; Δ, deletion; –, number of base pairs deleted (e.g., "−10" indicates 10 bp deleted).

zygotic division (see below). From three independent injections in which 18, 20, and 52 zygotes were transferred into four surrogate mothers, respectively, 10 of 23 founder (F_0) pups were black (Table 1). Since silent mutations in the "*A*" allele or mutations in the recessive "*a*" allele might occur without effects on coat color, we used F1 and R1 primers (Fig. 1D) to amplify the TALEN-Agouti target sequences from tail DNAs of F_0 mice and documented mutations in 16 of 23 pups (Fig. 1E and Table 1). Nucleotide sequencing revealed that most mutant alleles manifested deletions of 1 to 13 bp near the target site. Seven mice that lacked any detectable mutations were agouti colored, as expected (numbered by brown "Ag" fonts and designated "WT only" in Fig. 1E). Notably, the overall mutational frequency (70%) approached that observed with the same TALEN pair transfected into ES cells (83%, see the ES pilot studies in Materials and Methods).

Many founder mice are mosaic. Of 16 mice that carried *Agouti* mutations, 9 also yielded detectable wild-type sequences (denoted at the right margin of Fig. 1E). Although we expected that all 10

black mice (designated by black Ag numbers at the left in Fig. 1E) would exhibit reading frameshift or truncation mutations, this was not the case; mice Ag-16 and Ag-17 had in-frame deletions of 9 and 12 bp, respectively, and a 12-bp deletion was the only mutation detected in tail DNA of mouse Ag-14. Five other black mice (Ag-05, Ag-11, Ag-13, Ag-15, and Ag-18) yielded single frameshift deletions seemingly unaccompanied by wild-type or other mutated sequences. Because identical biallelic mutations are unexpected to occur at appreciable frequencies on two homologous chromosomes (32), we reasoned that larger TALEN-induced deletions extending beyond R1 or F1 primer binding sites would eliminate the possibility of amplifying such alleles. We therefore used more widely spaced primers (F-far and R-far in Fig. 1D) to screen for larger deletions. Notably, deletions of 410, 313, 245, and 212 bp (confirmed by nucleotide sequencing) were detected in mice Ag-01, Ag-12, Ag-13, and Ag-18, respectively (Fig. 1E; see Fig. S2 in the supplemental material). Although the presence of large deletions and the loss of F1 or R1 binding sites can explain why only single 1-bp deletion mutant alleles were detected in tail DNAs of Ag-13 and Ag-18, such a mechanism did not account for the failure to detect additional alleles by nucleotide sequencing of tail DNA in Ag-05, Ag-11, Ag-14, and Ag-15.

Sequencing of tail DNA of mouse Ag-01 yielded not only wild-type and mutant alleles (10-bp deletion) but also a large deletion of 410 bp; mouse Ag-12 exhibited two distinct frameshift mutations, as well as a 313-bp deletion (Fig. 1E; see Fig. S3 in the supplemental material). Notably, the presence of three different *Agouti* alleles in these founder mice argued that zygotes in the G_2 phase or their derived two-cell embryos may have undergone TALEN-induced mutations to yield mosaic pups.

To directly visualize the action of TALENs in embryos, zygotes were screened at 6 and 24 h after TALEN injection by staining with mouse antibodies to phosphoserine-239 of γH2AX, followed by Alexa Fluor 488-conjugated goat anti-mouse IgG to detect the foci of DNA damage (green). Fixed slides were then hybridized with a FISH probe using an Alexa Fluor 594-labeled BAC (red) directed to the *Agouti* locus. In two such experiments, examination of 87 zygotes 6 h after injection revealed unfused male and female haploid pronuclei, 34 of which (39%) exhibited colocalization of red and green signals in at least one pronucleus (see Fig. S3 in the supplemental material for an example). Because the first round of DNA replication begins even when discrete pronuclei are visualized (33), mosaic mutants can be generated in the late one-cell stage. Moreover, at 24 h postinjection after embryos reached the two-cell stage, 6 of 62 cells (~10%) yielded colocalized signals, implying that TALEN-induced cleavage continued, albeit less efficiently, after zygotes had undergone division. Hence, many F_0 animals are expected to be mosaic.

Inheritance of mutant *Agouti* alleles. To resolve the potential impact of *Agouti* mosaicism, as well as the confounding impact of large deletions that occurred in founder mice, representative F_0 animals were bred to C57BL/6 mice, and F_1 heterozygotes (containing one obligate wild-type *Agouti* allele) were genotyped to score germ line transmission of mutant alleles. Figure 2 shows the distribution of mutant *Agouti* sequences in 83 F_1 pups derived from seven F_0 animals. Two parental F_0 mice, Ag-06 and Ag-09, transmitted previously detected wild-type and mutant alleles through the germ line as expected. Founder Ag-01 transmitted both a mutant (10-bp deletion) and wild-type allele but did not transmit the allele with the larger 410-bp deletion to pups in this litter. The progeny derived from each of the four other F_0 mice inherited various *Agouti* alleles that had not been initially detected in the tail DNA of their parents. Mouse Ag-02 sired 11 pups, 4 of which contained an additional 8-bp deletion not previously seen in the F_0 parent. Mouse Ag-05, in which only a 4-bp deletion had been detected in the F_0 tail DNA, produced an equal number of mutant and wild-type progeny. All three mutations, including the large deletion detected in the tail DNA of founder Ag-12, as well as a fourth previously undetected wild-type allele, were distributed among its 18 F_1 pups. Finally, a black founder mouse, Ag-16, which paradoxically exhibited only an in-frame deletion of 9 bp in tail DNA, transmitted a 2-bp-frameshifted allele in lieu of the 9-bp-deleted allele to its F_1 progeny.

Several conclusions can be drawn from these findings, taken together. (i) All F_0 animals that exhibited *Agouti* mutations in tail DNA transmitted mutations through the germ line. (ii) However, additional and previously undetected mutant or wild-type alleles from mosaic F_0 mice were also inherited. (iii) In cases in which only a single mutant allele was detected in a founder mouse (e.g., Ag-05), the presence and subsequent transmission of a cryptic wild-type allele confirmed that interchromosomal recombination (gene conversion) did not occur in the founder. (iv) Black F_0 mice exhibiting only an in-frame deletion in tail DNA (e.g., Ag-16) carried cryptic frameshift mutations in the "*A*" allele that explained their altered coat color. (v) Finally, despite the fact that TALEN pairs flank defined "spacer" sequences of limited complexity, TALENs can generate much larger deletions which are heritable (also see below). These are most likely repaired via distal sites of microhomology (13).

Simultaneous gene disruption with multiple TALEN pairs. Previous studies revealed that embryoid bodies derived from ES cells lacking the *Arf* tumor suppressor gene are significantly retarded in generating extraembryonic endoderm but that this defect can be rescued by enforced expression of a single microRNA, miR-205 (28). Based on these observations, we constructed TALEN pairs targeting *miR-205* and *Arf* (see Fig. S1 in the supplemental material) and validated their ability to disrupt these two genes in ES cells. Biallelic inactivation of either gene resulted in a failure to properly generate extraembryonic endoderm (data not shown).

To determine whether different TALEN pairs might function simultaneously at relatively equal efficiency, we simultaneously injected TALENs targeting *Agouti* and *miR-205* into zygotes and derived 16 founder mice, 8 of which were black (Fig. 3). Thirteen of sixteen founder mice (81%) derived from two such experiments (Table 1) exhibited detectable mutations of both genes, two animals had mutations in either *Agouti* (AgMi-05) or *miR-205* (AgMi-13), and one had no mutations in either gene (AgMi-11). All black mice had *Agouti* frameshifts and/or large deletions, and the spectrum of mutations and their frequency mimicked what had been seen after injection of Agouti-TALENs alone (Fig. 1 and 3A). For unexplained reasons, mutations affecting pre-*miR-205* coding sequences tended to localize in the region complementary to the reverse TALEN as opposed to the spacer sequence (Fig. 3B).

To study germ line transmission of mutant alleles, seven representative AgMi F_0 mice were mated with wild-type C57BL/6 animals, and F_1 pups were genotyped as before using primers closely flanking the target sites, as well as distal primer pairs (F-far and R-far) directed to both targeted genes. As observed above,

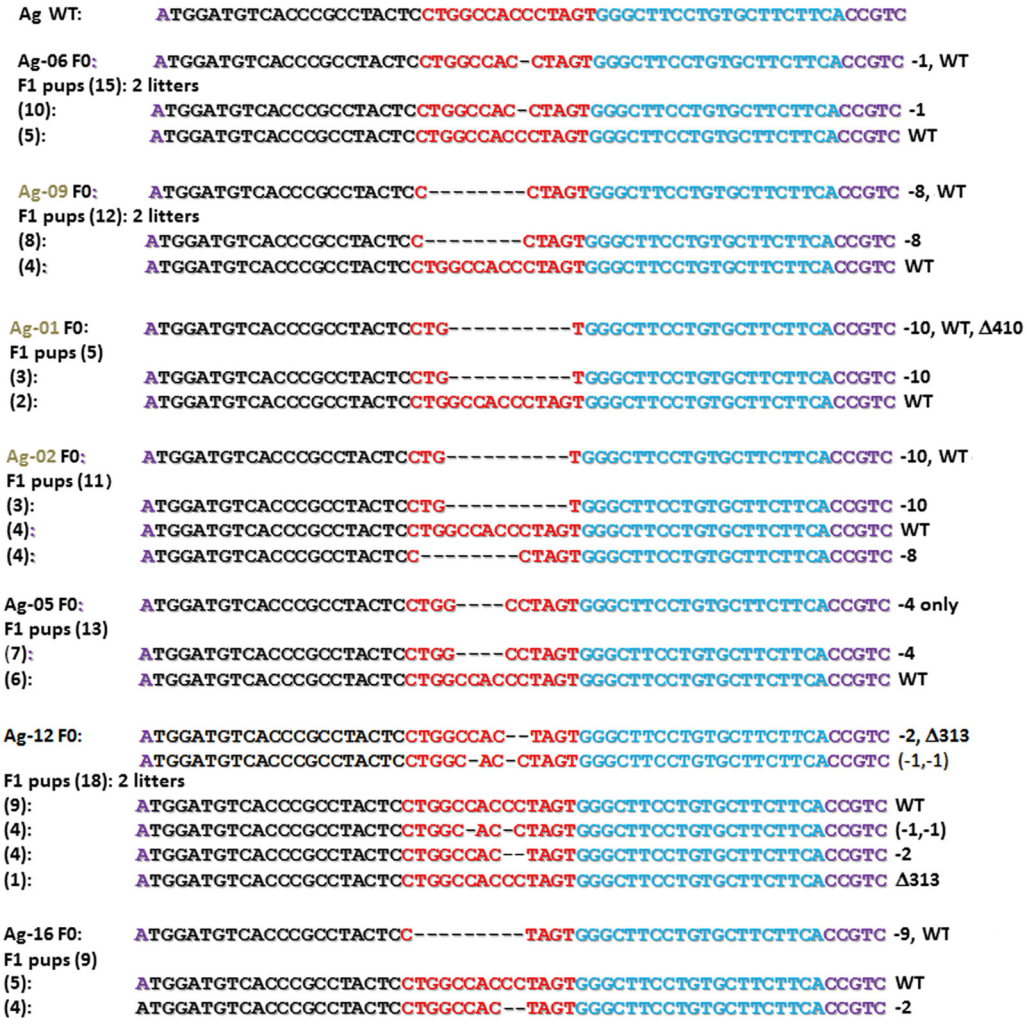

FIG 2 *Agouti* (Ag) genotypes of F_1 pups derived from breeding seven Ag F_0 mice to wild-type C57BL/6 mice. The nucleotide sequences of forward (black) and reverse (blue) TALEN binding sequences at the wild-type *Agouti* locus are shown at the top. Mutations detected in individual F_0 mice (top) and their respective F_1 offspring (bottom) are listed. Each Ag founder mouse (corresponding to those in Fig. 1) with the number of derived F_1 pups of indicated genotypes (parenthesis) are shown at the left. Mutated alleles summarized at the right (as for Fig. 1) include additional large deletions (Δ) detected using F-far and R-far primers (Fig. 1D) and confirmed by nucleotide sequencing. Black and brown F_0 mice are denoted by corresponding Ag font colors at the left. Symbols: WT, wild type; Δ, deletion; –, number of base pairs deleted (e.g., "−10" indicates 10 bp deleted).

nucleotide sequencing and deletion screening of the tail DNA of the founder mice only partially predicted the patterns of inheritance of mutant targeted alleles. Based on tail DNA sequences of F_1 pups, four of seven F_0 mice were mosaic for either *Agouti* or *miR-205* mutations, and three were mosaic for both (Table 2). Both small and large deletions were inherited. Five founder mice exhibited large deletions of *Agouti* ranging from 108 to 419 bp (Fig. 3A). One such black F_0 animal, AgMi-14, that exhibited two large *Agouti* deletions of 140 and 380 bp in tail DNA, transmitted these two mutated alleles independently to nine pups (Table 2; also see Fig. S4 in the supplemental material). Five of sixteen founder animals exhibited large *miR-205* deletions of 100 to 467 bp (Fig. 3B; also see Fig. S5A and B in the supplemental material), as again confirmed by nucleotide sequencing analysis. Although inactivation of *miR-205* in the germ line has been reported to induce strain-specific embryonic or early postnatal lethality (34, 35), mosaic *miR-205* mutant founders or F_1 heterozygotes derived from them remained healthy and without overt phenotypic anomalies. Notably, despite mosaicism and multigene targeting, the compound genotypes of F_0 mice were unraveled by backcrossing founders to wild-type animals.

Given the abilities of two TALEN pairs to act independently at equally high efficiency, we performed an additional larger-scale experiment in which 250 zygotes, injected with a mixture of TALEN pairs directed to *Agouti*, *miR-205*, and *Arf*, were distributed to 10 foster mothers. Of 43 pups obtained, the tail DNAs of 25, 36, and 24 F_0 mice were genotyped for *Agouti*, *miR-205*, and *Arf* mutations, respectively, pointing to efficient mutagenesis of all three genes (Table 1). Seven of 27 mice (29%) genotyped for *Arf* mutations also carried mutations of the other two genes (see Fig. S6 in the supplemental material) approximating the 24% frequency predicted from the product of individual mutational frequencies of the three genes (Table 1) and implying that the three TALEN pairs functioned independently without interference.

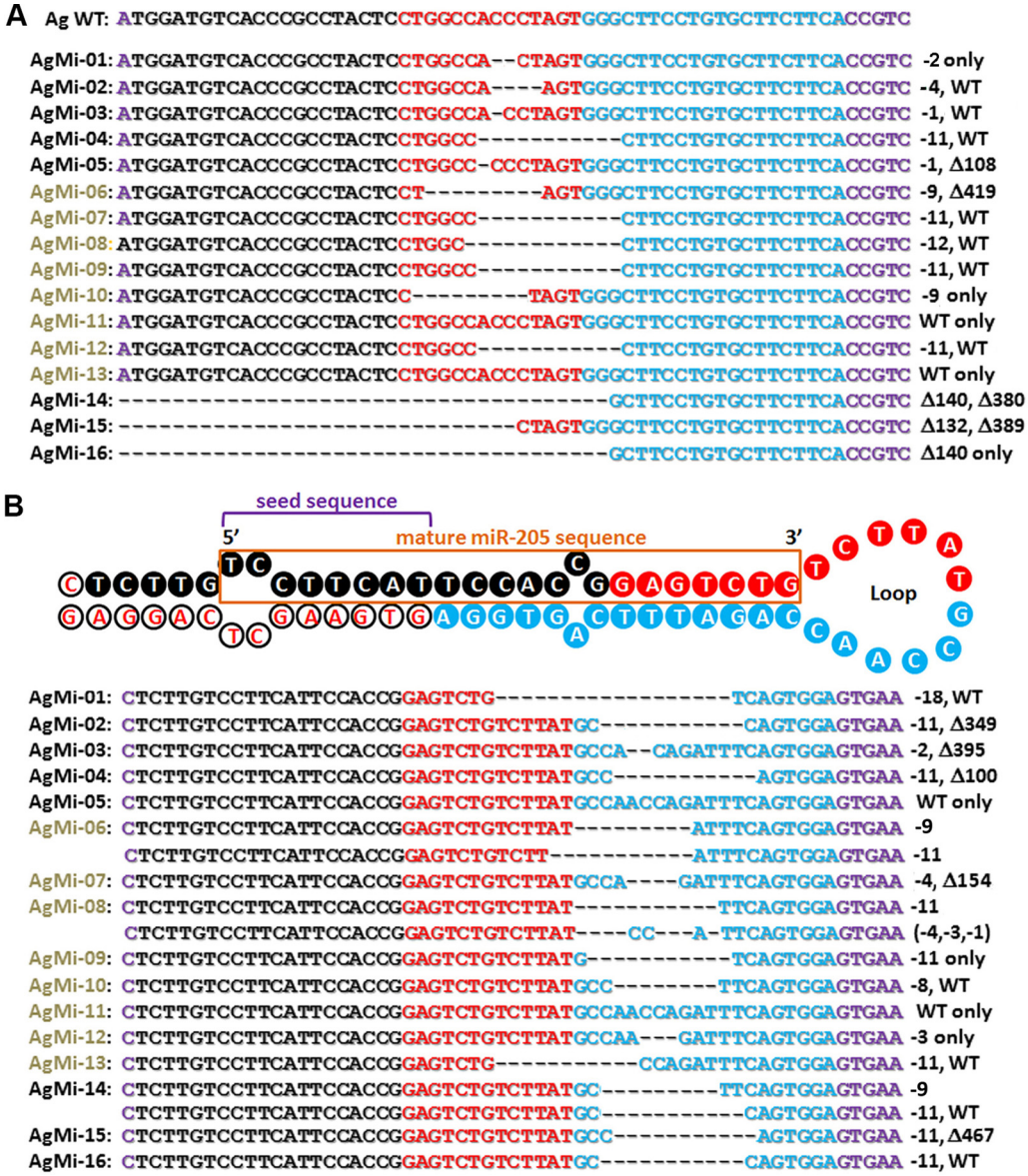

FIG 3 Nucleotide sequences of founder AgMi mice derived after zygotic coinjection of TALEN mRNA pairs directed to the *Agouti* (Ag) and *miR-205* (Mi) loci. The tail DNAs of individually numbered AgMi mice (left margin) were genotyped for Agouti (A) and miR-205 (B) mutations, summarized at the right as for Fig. 1. Sequences bound by forward (black) and reverse (blue) TALENs interrupted by spacer sequences (red) are color coded. The nucleotide sequence of pre-*miR-205* shown in panel B denotes one of two strands (orange box) of processed *miR-205*. Seed sequences are denoted by the purple bracket. Mutated alleles summarized at the right include additional large deletions (Δ). Black and brown F_0 mice are denoted by corresponding AgMi font colors. Symbols: WT, wild type; Δ, deletion; –, number of base pairs deleted (e.g., "−11" indicates 11 bp deleted).

DISCUSSION

Inactivation of selected targeted genes following inaccurate repair of DSBs by NHEJ can be readily achieved by introducing TALENs into cultured cells (4–6). The specificity of gene disruption depends on the use of customized TALEN pairs, each of which recognizes linked but separated genomic sequences of significant complexity (usually 20 bp, separated by 14 to 16 bp). Given the ability to assemble TALENs directed to virtually any sequence in the mouse genome, the selection of unique target binding sites is readily achieved, restricted only by a requirement for recognition of a T residue by the first TALE monomer. Given the high stringency of site-specific paired TALEN binding, and the requirement for FokI dimerization to initiate DNA cleavage, off-target effects in cultured cells are at best rare and frequently unobserved (14, 15, 36). Over the last year, several groups have injected TALEN mRNAs directly into fertilized one-cell embryos that, after their transfer to foster mothers, yielded founder mice carrying mutations in any one of several independently targeted genes (23–26). We adapted this strategy to simultaneously disrupt multiple genes, thereby generating founder mice that carry different combinations of heritable mutated alleles.

Microscopic two-color visualization revealed that TALEN mRNAs induced targeted DSBs by 6 h after injection into zygotes with 39% of 87 individually examined one-cell embryos exhibit-

TABLE 2 Simultaneous inheritance of *Agouti* and *miR-205* mutations in AgMi mice[a]

Founder mouse (color)	Founder (F_0) genotype		Total no. of F_1 pups	F_1 genotype mutation type (no.)	
	Agouti	*miR-205*		*Agouti*	*miR-205*
AgMi-01 (black)	–2 only	–18, WT	10	–2 (5), –2† (1), –29 (4)	–18 (7), WT (3)
AgMi-02 (black)	–4, WT	–11, Δ349	9	–4 (5), WT (4)	–11 (4), –18 (3)†, Δ349 (2)
AgMi-03 (black)	–1, WT	–2, Δ395	10	–1 (3), WT (5), –6 (2)†	–2 (4), Δ395 (3), –11 (3)†
AgMi-07 (agouti)	–11, WT	–4, Δ154	9	–11 (4), WT (2), –18 (3)†	–4 (2), –4 (4)†, Δ154 (3)
AgMi-10 (agouti)	–9 only	–8, WT	9	–9 (7), WT (2)†	–8 (8), WT (1)
AgMi-14 (black)	Δ140, Δ380	–9, –11, WT	9	Δ140 (4), Δ380 (5)	–9 (4), –11 (2), WT (3)
AgMi-15 (black)	Δ132, Δ389	–11, Δ467	12	Δ132 (5), Δ389 (2), WT (5)†	–11 (2), –4 (3)†, Δ467 (7)

[a] AgMi founder (F_0) mice were derived by coinjection of TALEN-Agouti and TALEN–miR-205 pairs into zygotes. Founder mice were mated to wild-type C57BL/6 mice and F_1 heterozygous pups, each containing at least one wild-type *Agouti* and *miR-205* parental allele (data not shown), were genotyped by nucleotide sequencing analysis and deletion screening of tail DNAs. The numbers of pups with the designated mutations are indicated in parentheses. The numbers of base pairs deleted are indicated by "–" or "Δ" (as noted in the figure legends). †, F_1 mouse alleles that were not detected in founder mouse tail DNA. WT, wild type.

ing confluent γH2AX foci at the *Agouti* locus in at least one pronucleus. At 24 h postinjection after the first cell division had occurred, 10% of cells yielded colocalized signals. Because chromosomes in microscopically visualized pronuclei have already entered their first S phase (33), TALENs can potentially target four *Agouti* alleles even before one-cell 4N embryos enter mitosis, implying that efficient gene disruption should frequently yield mosaic founder mice.

As predicted, breeding of TALEN-Agouti F_0 offspring to wild-type C57BL/6 mice produced litters of F_1 mice that contained as many as four distinct *Agouti* mutations. All seven Agouti-TALEN F_0 founders transmitted one or more mutant alleles to 58% of 83 offspring. Mutations detected in the tail DNAs of F_0 mice, including large deletions, were inherited through the germ line, but additional cryptic mutations that were not initially observed in F_0 mice were also revealed in the F_1 progeny. After simultaneous injection of TALEN pairs directed to both *Agouti* and *miR*-205, seven additional founder mice produced from zygotes were mosaic for one (4 mice) or both (3 mice) targeted genes, and all mutated alleles detected in F_0 mice (including large deletions), as well as cryptic mutant alleles, were inherited. Again, 72% of 68 offspring carried mutated *Agouti* alleles, and an even higher percentage inherited *miR-205* mutations. Using the same quantity of injected TALEN mRNA, one group reported a mutational efficiency for single genes of 77% (similar to that observed in our study), but only three F_0 mice were subsequently mated to produce F_1 offspring and mosaicism was rarely detected (25). In contrast, another group observed mosaicism in a majority of founders (24). Notably, the high frequency of mosaicism seen in our study argues against the utility of one-step mutagenesis in F_0 mice. Instead, establishment of defined mutant strains requires an additional round of breeding to produce F_1 progeny that together carry an allelic series of different mutations of the targeted genes.

The frequency of *Agouti* mutation observed in F_0 pups after simultaneous injection of two or even three TALEN pairs was equivalent to that obtained after targeting the *Agouti* locus alone. We did not fully analyze the 43 F_0 mice emanating from injection of three TALEN pairs, but for convenience, focused our initial attention on 14 black mice mutant for the *Agouti A* allele, seven of which also carried mutant miR-205 and *Arf* alleles. Because we routinely transferred ~20 injected zygotes into each foster mother, we cannot conclude that TALEN mRNA microinjection did not induce toxicity. However, our results suggest that injection of mRNAs encoding three pairs of TALENs was as well tolerated as the introduction of a single pair.

An alternate powerful technology for gene editing depends upon clustered regulatory interspaced short palindromic repeat (CRISPR)/Cas-based endonucleases. As presently configured, this system allows robust RNA-guided genome modifications using a single guide RNA that is designed to recognize 20-bp cDNA sequences via Watson-Crick base pairing. Target gene recognition also requires the presence of a "protospacer-adjacent motif" (PAM) in the targeting sequence, usually NGG, where N is any nucleotide (37–39). Like TALEN pairs, the CRISPR/Cas system induces DSBs within a target gene, which can be repaired by NHEJ or used to direct site-specific homologous recombination. Multiple guide RNAs encoded into a single CRISPR array (multiplexing) can enable simultaneous editing of several sites within mammalian genomes (40–42) and, indeed, coinjection of Cas9 and guide RNAs into zygotes generated founder mice with biallelic mutations in both the *Tet1* and *Tet2* genes (43), although mosaicism was not assessed.

The relative merits of TALEN and CRISPR/Cas-based strategies for gene editing require further assessment. It has been widely assumed that CRISPR/Cas may provide a more efficient platform for gene disruption, but recent work with TALENs in ES cells and zygotes suggests otherwise. Generating multiple CRISPR/Cas guide RNAs is much less arduous than synthesizing TALEN pairs for multiple genes, although newly developed automated methods for TALEN assembly show much greater promise in facilitating high-throughput gene editing (19–22). One limitation of CRISPR/Cas is the requirement for PAM motifs at the target site, whereas TALENs require only a 5′ T residue. Despite this seemingly small difference, PAM motifs may not exist within small genes, such as some microRNAs, limiting the effective use of CRISPR/Cas at these loci. Regardless of which of these technologies is applied, the specificity of the designer nucleases is crucial in avoiding undesired off-target cleavages that may go unrecognized and that might potentially contribute to phenotypic changes. The key to eliminating off-target effects is the choice of unique target sites within the genome (22, 44). The more stringent recognition sequences of TALEN pairs versus the lower complexity of CRISPR/Cas guide RNAs, and the tolerance of the latter system for single base mismatches, suggest that CRISPR/Cas may induce more off-target effects (40, 44, 45). However, paired reengineered Cas9 nickases (working in a manner analogous to TALEN pairs) coupled with optimal guide RNA design should improve specificity (38, 44, 46). Finally, reengineering of Cas9 to create a catalytically inactive RNA-dependent DNA-binding protein provides a powerful new system for regulating gene expression (47), and it is

unclear whether TALENs might afford an equally flexible platform.

Since TALENs and CRISPR/Cas generate site-specific DSBs, both strategies are expected to facilitate gene editing by homologous recombination using coinjected oligonucleotides or conventional targeting plasmids. Although we have not attempted to inject conventional targeting constructs together with TALENs into zygotes in order to direct homologous recombination at targeted genes, we have successfully used this strategy to create "knockin" *Arf* alleles in ~5% of ES clones tested (see the discussion of pilot studies with ES cells in Materials and Methods). Application of either technology in zygotes provides an efficient strategy for simultaneously generating a spectrum of multi-allelic inactivating mutations in founder mice and for bypassing traditional time-consuming steps in gene editing technology.

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